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Conidial morphology changes in four *Phyllosticta* species

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Abstract — Production of pycnidia and conidia by *Phyllosticta theacearum*, *Phyllosticta* sp. (ex *Euonymus*) and *Phyllosticta* sp. (ex *Portulaca*) (grown on PDA), and *P. cruenta* (from specimens) was studied. On PDA, conidial appendages of *Phyllosticta* sp. (ex *Portulaca*) disappeared completely after 16 days, of *P. theacearum* after 29 days, and of *Phyllosticta* sp. (ex *Euonymus*) after 14 days. In slide preparations mounted in water, appendages of all four species persisted; the appendages of *P. cruenta* and *Phyllosticta* sp. (ex *Euonymus*) became 2–4 times longer within 30 minutes, while those of *Phyllosticta* sp. (ex *Portulaca*) and *P. theacearum* remained unchanged.

Key words — caducous, extend, identification, anamorph, *Ascomycota*, *Phoma*

Introduction

Species of the anamorphic ascomycete genus *Phyllosticta* Pers. have often been recorded as plant pathogens, saprobes, and endobionts (Liu & Lu 2007, Okane et al. 2003, Punithalingam 1974). Identification is highly problematic, as few characters are available to separate different species. Sometimes a single difference in conidial morphology, such as thickness of the mucoid layer surrounding the conidium or appendage shape or size, has been used to distinguish different species (Motohashi et al. 2008a,b, Nag Raj 1993, Punithalingam & Woodhams 1982, Aa & Vanev 2002, Wulandari et al. 2009). There have also been frequent confusions between *Phyllosticta* and *Phoma,* which have many morphological similarities. The key distinctions between these two genera are the mucoid sheath and appendage, reported for *Phyllosticta* but not for *Phoma*. Those two structures are, however, delicate, faint, colourless, and easily overlooked under the poorly contrasting conditions of bright field microscopy. In the past, that has made misidentifications between *Phyllosticta* and *Phoma* frequent. With improvements in the quality of microscopes and the wide availability of phase contrast and differential interference contrast optics, it is now easier to detect mucoid sheaths and appendages. But there is

an additional problem: in some species of *Phyllosticta* the appendages can be caducous even in fresh material and cultures (Aa 1973).

The objectives of the present paper are therefore to report preliminary observations about the time span during which appendages can be observed, and to record some changes that can occur in appearance of appendages during that period. As the species used in this study are not well documented, the opportunity is also taken to provide illustrations and descriptions of appearances in pure culture.

Materials & methods

Isolates were obtained using standard phytopathological techniques, and for three species pure cultures were established (*P. cruenta* failed because of contamination by bacteria) and incubated at 28°C in the dark. Slants on PDA were preserved at 4°C in a refrigerator. Fungal identifications were based on specific literature such as the contributions as Aa (1973), Aa & Vanev (2002) and Nag Raj (1993) among others. All slides were prepared using sterile technique. Observations of pycnidia and conidia were made on subcultures derived from original slants. Starting from 5 days after making the subculture, each Petri dish was examined daily for pycnidia. When pycnidia were detected, a slide was prepared to check for presence of conidia. The date of first appearance of conidia was then noted. From first appearance of conidia, slides were prepared daily to observe appendages. Two key dates were noted: that on which at least some conidia no longer had appendages, and that on which no conidia remained with visible appendages. Thereafter observations were discontinued. Squash mounts of pycnidia were also prepared in water to observe conidial appendages. Each slide prepared in this way was then examined at three minutes intervals for 30 minutes, and the condition of appendages noted and photographed using bright field, phase contrast, and differential interference contrast microscopy. Where no change in appendage appearance was noted, the slide was then placed on moist filter paper in a Petri dish and re-observed every 30 minutes up to 3 hours. Material that still showed no change was then re-observed daily for three days.

Specimens examined – *Phyllosticta cruenta* (Fr.) J. Kickx f.: **CHINA, Shandong**: Kunyu Mountain, in diseased leaves of *Polygonatum odoratum* var. *pluriflorum* (Miq.) Ohwi (*Convallariaceae*), 37°17'04.76"N, 121°44'49.24"E, 284 m, 12 July 2007, MHQAU0134.

Phyllosticta theacearum Aa: **CHINA, Shandong**: Laoshan Mountain, in diseased leaves of *Thea sinensis* L. (*Theaceae*), 36°11'00.42"N, 120°40'49.50"E, 79 m, 25 April 2008, MHQAU0192, JJ196.

Phyllosticta sp. (*Euonymus*): **CHINA, Shandong**: campus of Qingdao Agricultural University, in diseased leaves of *Euonymus japonicus* Thunb. (*Celastraceae*), 36°19'16.50"N, 120°24'01.62"E, 9 m, 22 July 2009, MHQAU0248, JJ243.

Phyllosticta sp. (*Portulaca*): **CHINA, Shandong**: campus of Qingdao Agricultural University, as endobiont from stem of *Portulaca oleracea* L. (*Portulacaceae*), 36°19'11.91"N, 120°23'58.18"E, 9 m, 19 July 2008, JJ219.

All the specimens and isolates are deposited in Mycology Herbarium, Qingdao Agricultural University (MHQAU).

Results

Appearance of cultures on PDA

Phyllosticta theacearum: mycelium immersed and superficial, initially pale grey, gradually turning greenish black, dense, floccose, margin undulate with abundant submerged mycelium (Fig. 1A); reverse black (Fig. 1B).

Phyllosticta sp. (*Euonymus*): colonies were initially pale yellow, eventually turning grey with thin submerged mycelium; aerial mycelium scanty, sparse, thin; margin irregular with sparse submerged mycelium (Fig. 1N); reverse with a grey centre surrounded by a black ring and a grey perimeter (Fig. 1O).

Phyllosticta sp. (*Portulaca*): mycelium immersed and superficial, white at first, becoming greyish black; surface covered by a thin mat of whitish aerial mycelium, dense, flat, fissured, with a clearly-defined edge (Fig. 1R); reverse greyish black (Fig. 1S).

Production of pycnidia and conidia on PDA

Phyllosticta sp. (*Portulaca*) produced pycnidia and conidia in 8 days; *Phyllosticta* sp. (*Euonymus*) produced pycnidia and conidia in 7–8 days; *P. theacearum* produced pycnidia and conidia in 14 days.

Loss of appendages with time

Appendages of *P. theacearum*, *Phyllosticta* sp. (*Euonymus*), and *Phyllosticta* sp. (*Portulaca*) disappeared with ageing of colonies on PDA. Conidia of *Phyllosticta* sp. (*Portulaca*) with no visible appendages began to be observed 5 days after conidia were first sighted, and by 8 days no appendages could be seen. Conidia of *Phyllosticta* sp. (*Euonymus*) with no visible appendages began to be observed 3–4 days after conidia were first sighted, and by 6 days no appendages could be seen. Conidia of *P. theacearum* with no visible appendages began to be observed 10 days after conidia were first sighted, and by 15 days no appendages could be seen.

Changes in appearance of appendages in water

Appendages of *Phyllosticta* sp. (*Portulaca*) (Fig. 1T) and *P. cruenta* (Fig. 1G, H) were initially about 50% of the length of conidia. Appendages of *Phyllosticta* sp. (*Euonymus*) (Fig. 1P) and *P. theacearum* (Fig. 1C) were initially between 50% and 100% of the length of conidia. In water mount slides, appendages of *P. cruenta* and *Phyllosticta* sp. (*Euonymus*) extended, while those of *Phyllosticta* sp. (*Portulaca*) (Fig. 1T) and *P. theacearum* (Fig. 1D) remained unchanged, retaining their original length even in water for three days. Appendages of *P. cruenta* began to extend after 6 minutes in water (Fig. 1I) and became 3–4 times the original length in 18–30 min (Fig. 1J, K, arrows). After that, no further extension occurred. In *P. cruenta*, the conidia contained small greenish guttules (Fig. 1G–H) which, with time immersed in water, fused into two large drops (Fig. 1J, K). Guttules in the other three species did not fuse. Appendages

of *Phyllosticta* sp. (*Euonymus*) extended to 2–3 times their original length in 12–30 min (Fig. 1Q, arrow) and no change occurred thereafter.

Discussion

The presence or absence of appendages is routinely used to distinguish *Phyllosticta* species (Nag Raj 1983, Punithalingam & Woodhams 1982, Aa 1973, Weidemann et al. 1982). Aa (1973), however, reported that *Phyllosticta* appendages may be caducous in freshly collected specimens and even in pure cultures, being usually visible only on a proportion of the conidia. For cultures on PDA, the present results confirm that appendages of some species in *Phyllosticta* can disappear with time. Prior to the present study, apparently nothing was known about *Phyllosticta* appendage longevity on artificial media. For all three *Phyllosticta* species studied here on PDA, the longest period of appendage visibility lasted no longer than one month. Earlier reports of *Phyllosticta* species lacking appendages may need to be reviewed in the light of this result. Longevity of appendages in cultures on other artificial media commonly used to grow *Phyllosticta* species also needs to be determined, and it may be advisable to record the age of cultures used when describing *Phyllosticta* appendages.

When mounted in water, appendages of the four species of *Phyllosticta* in the present study remained persistent, but responded differently. Those of *P. theacearum* and *Phyllosticta* sp. (*Portulaca*) kept their original length in water for several days, while those *P. cruenta* and *Phyllosticta* sp. (*Euonymus*) extended 2–4 times their original length in a short time (12–30 minutes). This phenomenon may explain why in earlier reports there is sometimes considerable divergence in the stated length of conidial appendages in the same species. Aa (1973), for example, reported that conidia of *P. vaccinii* were 8–12 × 5–8 µm and had appendages usually 4–8 µm long and sometimes up to 17 µm long, while Weidemann et al. (1982), studying the same species, reported larger measurements of spore appendage lengths. They described appendage lengths as ranging from 3–70 µm for conidia 8–13 × 7–9 µm in size. In another species *P. elongata*, Weidemann et al. (1982) reported appendages varying in length from 4–120 µm. Unfortunately, the time elapsed between preparing the slide and measuring the appendages was not stated. Earlier reports of appendage lengths should therefore be treated with caution. At present it is not clear if this phenomenon of extending appendages is widespread in *Phyllosticta*, or present only in some species. Given its potential impact on *Phyllosticta* identification, more species could usefully be studied.

The identity of the *Phyllosticta* spp. on *Euonymus japonicus* and on *Portulaca oleracea* merits discussion. Numerous taxa described as *Phyllosticta* spp. from *Euonymus* belong in other genera or are of dubious application (Aa & Vanev

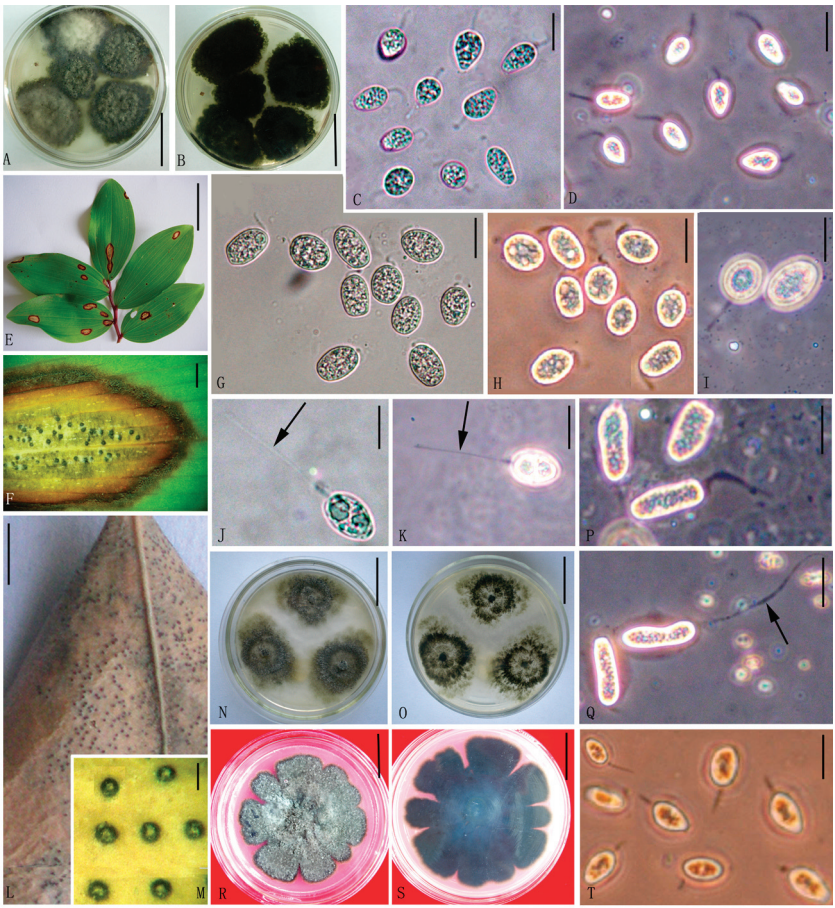

Plate 1 Four species of *Phyllosticta*. ***Phyllosticta theacearum***: (A) upper colony; (B) converse colony; (C) conidia; (D) conidia after three days in water. ***Phyllosticta cruenta***: (E) symptom on the leaf of *Polygonatum odoratum* var. *pluriflorum*; (F) pycnidia; (G,H) conidia; (I) conidia after 6 min. in water; (J,K) conidia after 18–30 min. in water. ***Phyllosticta* sp.** (on *Euonymus*): (L,M) pycnidia on the leaf of *Euonymus japonicus*; (N) upper colony; (O) converse colony; (P) conidia; (Q) conidia after 12–30 min. in water. ***Phyllosticta* sp.** (on *Portulaca*): (R) upper colony; (S) converse colony; (T) conidia. (C,G,J under light field; D,H,I,K,P,Q,T under phase contrast field.) Scale bars: A,B,E,L,N,O,R,S = 2.5 cm; F = 1 mm; M = 100 μm; C,D,G–K,P,Q,T = 10 μm.

2002). *Phyllosticta euonymi-japonici* L.L. Liu & G.Z. Lu is apparently the only species of *Phyllosticta* sensu stricto described from *Euonymus*; it differs from the present species in shape and dimension of conidia. The conidia of *P. euonymi-japonici* are ellipsoid to ovoid, 10–12.5 × 7.5–10 μm, while those of

Phyllosticta sp. (*Euonymus*) are clavate and 15–23 × 6–8 µm. It therefore seems possible that the present species is undescribed. *Phyllosticta* sp. (*Portulaca*) may also represent an undescribed species. There is no named species of *Phyllosticta* recorded on *Portulaca*, but Batista & Vital (1952: 57) reported *Phyllosticta* sp. on *P. oleracea* from Brazil. There is insufficient information to determine whether the Brazilian fungus is a species of *Phyllosticta* sensu stricto, or to compare it with our *Portulaca* isolate.

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